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Binding of [^{14}C]Erythromycin to *Escherichia coli* Ribosomes

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Erythromycin binding to *Escherichia coli* ribosomes required K^+ and Mg^{2+}. Under optimal conditions, the dissociation constant for erythromycin binding to *E. coli* ribosomes was found to be 1.0×10^{-8} M and 1.4×10^{-8} M at 24 C and 5 C, respectively. One molecule of [^{14}C]erythromycin was bound to each 70*S* ribosome at equilibrium. Binding of erythromycin to ribosomes was rapid and reversible. The specific rate constants for the forward and reverse reactions were 1.7×10^7 liters per mol per min and 0.15 per min, respectively.

Erythromycin, a macrolide antibiotic, interferes with bacterial growth by inhibition of protein synthesis (9). Although the precise mechanism of inhibition of protein synthesis by erythromycin has not been delineated, the inhibition of protein synthesis results from its ability to bind tightly to the 50*S* subunit of bacterial ribosomes. It was thus of interest to examine this binding in detail.

Erythromycin has been reported to bind to ribosomes from various species of bacteria (1, 4, 5, 7, 10, 11, 13). This communication describes the quantitative characteristics of erythromycin binding to *Escherichia coli* ribosomes. Specifically, the association constant of the erythromycin-ribosome complex was determined as well as the stoichiometry and requirements of the reaction.

MATERIALS AND METHODS

Materials. Ammonium chloride-washed *E. coli* 70*S* ribosomes and cell extracts were prepared as described previously (8). [^{14}C]erythromycin was synthesized by the method of Flynn et al. (2). The final erythromycin product had a specific activity of 45.7 mCi/mmol. It appeared as a single major spot on chromatography in two thin-layer chromatographic systems (6). Eastman thin-layer silica gel chromatogram sheets (no. 6061) were used with either carbon-tetrachloride-ethanol-dimethylformamide (7:2:1) or chloroform-methanol-dimethylformamide (24:90:6). Radioactive spots on the thin-layer chromatograms were determined by autoradiography. Electrophoresis was performed for 2.5 h in 10% (vol/vol) pyridine adjusted to pH 6.5 with acetic acid on Whatman 3MM paper and a voltage gradient of 74 V/cm (1). One major peak contained 91% of the radioactivity and corresponded to erythromycin, and a second slower moving peak contained 9% of the radioactivity (1). Microbiological assay of the [^{14}C]erythromycin was essentially equivalent to that of an unlabeled erythromycin A standard.

Determination of [^{14}C]erythromycin binding to ribosomes by filtration. Binding of [^{14}C]erythromycin to ribosomes was determined by a modication of the procedure described by Mao and Putterman (5). Each reaction mixture for determination of binding of [^{14}C]erythromycin to ribosomes contained the following components in a volume of 0.50 ml unless otherwise specified: 0.004 M $MgCl_2$; 0.1 M KCl; 0.01 M NH_4Cl; 0.01 M tris(hydroxymethyl) aminomethane (Tris)-chloride, pH 7.2; about 5.6 to 7.5 absorbance units of NH_4Cl-washed *E. coli* B or *E. coli* A19 ribosomes at 260 nm (A_{260}); and about 1.2 μM [^{14}C]erythromycin A. Reactions were started by adding ribosomes last to the reaction mixtures. Incubations were performed at 24 C for 30 min. At the end of the incubation, reactions were stopped by diluting the reaction mixture with 3 ml of cold solution A (0.005 M $MgCl_2$, 0.15 M KCl, and 0.01 M Tris-chloride, pH 7.2). The diluted reaction mixture was filtered through a 25-mm diameter membrane filter (0.45-μm pore size; HAWP, Millipore Corp.); the tube and filter were immediately washed an additional three times with 3 ml of cold solution A. The filters were then dried under an infrared lamp, and radioactivity was determined in a scintillation spectrometer as previously described (8).

Equilibrium dialysis. Reaction mixtures contained 0.004 M $MgCl_2$, 0.1 M KCl, 0.0025 M NH_4Cl, and 0.01 M Tris-chloride, pH 7.2 (solution B). Dialysis was performed in glass test tubes (13 by 100 mm) containing 1.5 ml of solution B. Ribosomes (137 pmol, 5.6 A_{260} units) were resuspended in 0.5 ml of solution B containing various concentrations of [^{14}C]erythromycin and placed within small dialysis tubes sealed with plastic caps and retaining rings (Pope Scientific Co., Menomonee Falls, Wisc.). The test tubes were covered and placed on a rotary shaker at 5 C for 70 h. Time for attaining equilibrium was determined by measuring [^{14}C]erythromycin concentration inside and outside of the dialysis membranes containing no ribosomes. Equilibrium was attained in about 48 h. Binding of antibiotic was determined by counting portions from both inside and outside the dialysis membrane. The difference in radioactivity

between the inside and the outside of the dialysis sac was taken as a measure of [^{14}C]erythromycin bound to ribosomes.

RESULTS

Effect of K^+ and Mg^{2+} on [^{14}C]erythromycin binding to ribosomes. The requirement for K^+ for [^{14}C]erythromycin binding to ribosomes is shown in Fig. 1. [^{14}C]erythromycin binding was negligible in the absence of K^+. At 100 mM K^+, [^{14}C]erythromycin binding was almost maximal. Although a minimal concentration of Mg^{2+} was required for optimal binding, at 0.1 mM Mg^{2+} (the lowest concentration tested) substantial [^{14}C]erythromycin binding to ribosomes was observed. Essentially, an identical magnesium curve was obtained with purified 50*S* subunits. Binding was maximal from 1 to 10 mM Mg^{2+}. Similarly, others have previously reported a monovalent cation requirement (K^+ or NH_4^+) for erythromycin binding to various bacterial species (5, 7, 12).

Binding of [^{14}C]erythromycin to ribosomes: filter assay and equilibrium dialysis. [^{14}C]erythromycin was very strongly bound to ribosomes. The [^{14}C]erythromycin-ribosome complex can be conveniently measured with the use of membrane filters (Fig. 2). In the presence of 5.6 A_{260} units (137 pmol) of ribosomes, 98% of the ribosomes bound a molecule of [^{14}C]erythromycin at an initial concentration of 9.5×10^{-7} M [^{14}C]erythromycin at 24 C. The results of equilibrium dialysis under comparable conditions, except that temperature was maintained at 5 C for 70 h, are also shown in Fig. 2. Association constants determined by filtration at 24 C and equilibrium dialysis at 5 C were 9.9×10^7 and 7.2×10^7 M^{-1}, respectively, as determined from the Scatchard plots (Fig. 2, insets). In addition, a value of 0.98 and 0.86 for the number of erythromycin molecules per ribosome was determined by filtration and equilibrium dialysis, respectively. The lower value of 0.86 may be due to some inactivation of the ribosomes during the 70 h of dialysis. A preliminary dialysis experiment performed for 46 h indicated that 91% of the ribosomes were capable of binding a molecule of [^{14}C]erythromycin. This compares to a value of 86% for the 70-h dialysis. This would be consistent with an inactivation rate of about 5% per day during the dialysis of ribosomes.

Comparison of filtration and equilibrium dialysis. To compare directly results of filtration and equilibrium dialysis, equilibrium dialysis was performed for about 70 h at 5 C. Samples from both the inside and outside of the dialysis sacs were taken for both total counts and for filtration. The [^{14}C]erythromycin bound to ribosomes as a function of [^{14}C]erythromycin concentration is shown in Fig. 3 for both assays. It can be seen that the filtration assay yields consistently less binding than does equilibrium dialysis. This is consistent with a slight loss of radioactivity during the washing procedure. Indeed, it was shown that continued washing of the [^{14}C]erythromycin-ribosome complex bound to filters resulted in loss of retained radioactivity; by washing with several liters of solution A, most of the radioactivity could be removed from the filters. Also, 1,000-fold dilutions of the reaction mixtures prior to filtration reduced binding to filters greatly, as would be expected if the binding was reversible (data not presented).

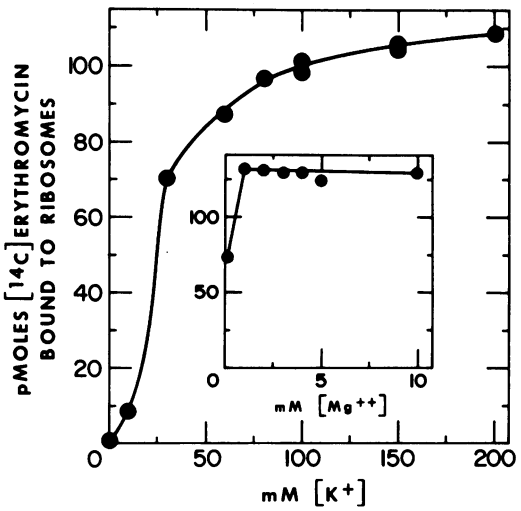

FIG. 1. *Effect of K^+ and Mg^{2+} on [^{14}C]erythromycin binding to ribosomes. Each 0.50-ml reaction mixture for the K^+ curve contained the following components: 0.004 M $MgCl_2$; 0.01 M Tris-chloride, pH 7.2; KCl as indicated on the abscissa; 1.1 μM [^{14}C]erythromycin A; and 6.4 A_{260} units of NH_4Cl-washed ribosomes. For the Mg^{2+} curve, each 0.50-ml reaction mixture contained the following components: 0.10 M KCl; 0.01 M NH_4Cl; 0.01 M Tris-chloride, pH 7.2; $MgCl_2$ as indicated on the abscissa of the inset; 1.1 μM [^{14}C]erythromycin A; and 7.5 A_{260} units of NH_4Cl-washed ribosomes. All reaction mixtures were incubated at 24 C for 30 min. Assays were performed as described.*

Reversibility of [^{14}C]erythromycin binding to ribosomes. If excess unlabeled erythromycin was added to reaction mixtures after formation of the [^{14}C]erythromycin-ribosome complex, radioactivity was rapidly lost from the complex (Fig. 4). Therefore, the binding of [^{14}C]eryth-

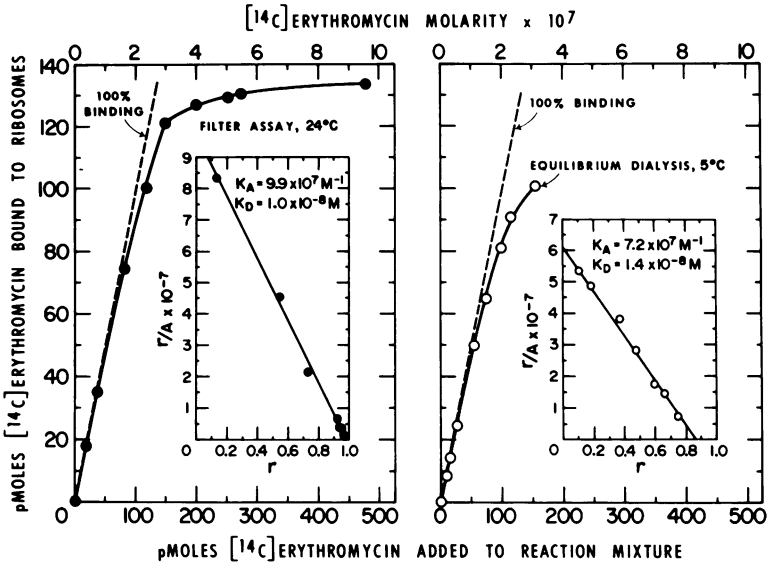

FIG. 2. *Binding of [14C]erythromycin to ribosomes as a function of [14C]erythromycin concentration. Each 0.50-ml reaction mixture for the filter assay (left panel) contained 5.6 A_{260} units of ribosomes and other components as described. The initial concentration of [14C]erythromycin added to the reaction mixture is given on the abscissa. Dissociation (K_d = 1.0 × 10^{-8} M) and association (K_a = 9.9 × 10^7 M^{-1}) constants were computed from the Scatchard plot (inset). Equilibrium dialysis (right panel) was performed, as described, at 46 and 70 h. The values at 70 are plotted directly and as a Scatchard plot (inset). At 46 h, the slope of the Scatchard plot was similar, but the baseline intercept was 0.91. At 70 h, the baseline intercept was 0.86. The dissociation (K_d = 1.4 × 10^{-8} M) and association (K_a = 7.2 × 10^7 M^{-1}) constants were calculated from the Scatchard plot. A computer program was used to determine the line of best fit by the method of least squares. The data for the filter assay are presented as solid circles (●) and the data for equilibrium dialysis by unfilled circles (○).*

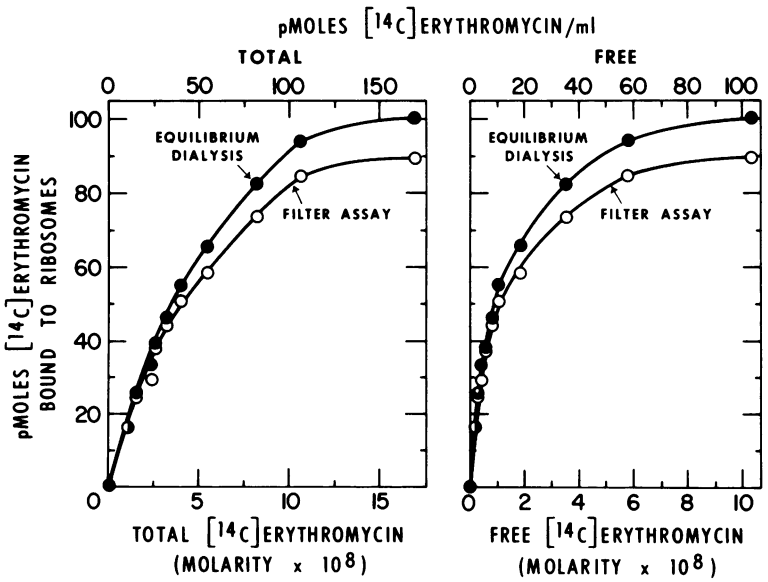

FIG. 3. *Comparison of filtration assay and equilibrium dialysis. Equilibrium dialysis was performed as described at 5 C for 70 h. At 70 h, duplicate 0.20-ml samples were taken from inside and outside the dialysis sacs. One sample was placed directly into a scintillation vial for determination of radioactivity. The other sample was filtered and washed as described for the filtration assay. The picomoles of [14C]erythromycin bound to ribosomes as a function of the total (left panel) or free (right panel) [14C]erythromycin concentration are presented in the figure. Symbols: ●, equilibrium dialysis; ○, filtration assay.*

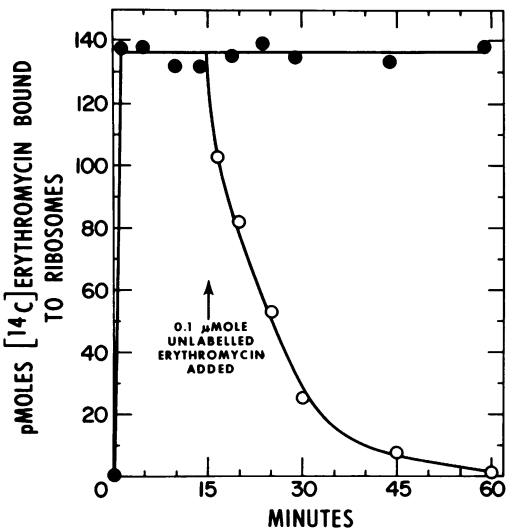

FIG. 4. *Reversibility of [14C]erythromycin binding to ribosomes. Each 0.50-ml reaction mixture contained 5.6 A_{260} units of ribosomes and other components as described. The time course of [14C]erythromycin binding to ribosomes was followed as a function of time at 24 C. At 15 min, 0.010 ml of a 0.01 M solution of unlabeled erythromycin was added to some of the tubes as indicated to produce a ratio of 200/1 for unlabeled to 14C-labeled erythromycin in these tubes. Symbols: ●, no unlabeled erythromycin; ○, 0.1 μmol of unlabeled erythromycin added to each of these tubes at 15 min.*

romycin to *E. coli* ribosomes is reversible as previously shown for ribosomes from *Bacillus subtilis* (7). The equation for the reaction is

$$E + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot R$$

where E, R, and E·R represent erythromycin, ribosomes, and the erythromycin-ribosome complex, respectively, The specific rate constant for the reverse reaction (k_{-1}) was calculated from the data of Fig. 4 to be 0.15 per min. Although the rate of binding of [14C]erythromycin to ribosomes was too fast to be measured, since $K_a = k_1/k_{-1}$, the specific rate constant for the forward reaction could be computed ($k_1 = K_a \cdot k_{-1}$); k_1 was calculated to be 1.5×10^7 liters per mol per min. A summary of these parameters is presented in Table 1.

Does [14C]erythromycin bind to ribosomal ribonucleic acid? The binding of [14C]erythromycin to ribosomal ribonucleic acid from *E. coli* was examined by equilibrium dialysis as described above. In the presence of 24 A_{260} units of ribosomal ribonucleic acid in the 0.5 ml inside the dialysis sac, no binding of [14C]erythromycin was detectable (data not shown).

DISCUSSION

The present results confirm that both Mg^{2+} and K^+ are required for optimal binding of erythromycin to *E. coli* ribosomes as previously indicated for other bacterial ribosomes (5, 7). In addition, a comparison of the filtration assay and equilibrium dialysis indicated that there was a slight loss of [14C]erythromycin from the [14C]erythromycin-ribosome complex bound to the filter during the washing procedure. This loss was relatively small, amounting to about 10% or less of the [14C]erythromycin bound to ribosomes.

The association constant for the binding of [14C]erythromycin to ribosomes at 5 C was determined to be $7.2 \times 10^7\ M^{-1}$, as determined by equilibrium dialysis. Since equilibrium dialysis could not be performed at 24 C for a short time, the association constant at 24 C was estimated by the filtration assay. The association constant thus determined at 24 C was $9.9 \times 10^7\ M^{-1}$. These association constants are significantly higher than the association constants of 3 to $5 \times 10^5\ M^{-1}$ and $9.2 \times 10^6\ M^{-1}$, which were reported for [14C]erythromycin binding to ribosomes from *Staphylococcus aureus* (5) and *E. coli* (1), respectively. It is likely that the association constant reported for the *S. aureus* ribosomes was significantly underestimated by at least two orders of magnitude.

The present results allow us to use [14C] erythromycin binding to *E. coli* ribosomes as an assay for active analogues of erythromycin. In addition, having an accurate value for the association constant for the erythromycin-ribosome complex, we should be able to determine the association constant of each erythromycin analogue relatively accurately using the method of Harris and Pestka (3). We evaluated the binding of various erythromycin analogues for several purposes. We are correlating the effect of these analogues on binding of erythromycin to ribosomes with their antibacterial activity. Furthermore, we wish to determine

TABLE 1. *Kinetic and equilibrium constants for [14C]erythromycin binding to E. coli ribosomes at 24 C*

Constant[a]	Value
K_a	$9.9 \times 10^7\ M^{-1}$
K_d	1.01×10^{-8} M
k_1	1.5×10^7 liters per mole per min
k_{-1}	0.15 per min

[a] K_a, Association constant; K_d, dissociation constant; k_1, absolute rate constant for the forward reaction; k_{-1}, absolute rate constant for the reverse reaction.

which areas of the erythromycin molecule can be modified with retention of activity so that we can attach reactive groups to these sites. These modified erythromycin analogues will provide a basis for study of the topology of the erythromycin bindng site by affinity labeling of the proteins or nucleic acids which comprise this site. In the accompanying communication, the effect of a number of erythromycin A analogues on [^{14}C]erythromycin binding to ribosomes is presented.

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